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Abstract: BACKGROUND: Influenza vaccine immunogenicity is suboptimal in immunocompromised patients. However, there are limited data on the interplay of T- and B- cell responses to vaccination with simultaneous immunosuppression. METHODS: We collected peripheral blood mononuclear cells from transplant recipients before and 1 month after seasonal influenza vaccination. Before and after vaccination, H1N1-specific T- and B-cell activation were quantified with flow cytometry. We also developed a mathematical model using T- and B-cell markers and mycophenolate mofetil (MMF) dosage. RESULTS: In the 47 patients analyzed, seroconversion to H1N1 antigen was demonstrated in 34%. H1N1-specific interleukin 4 (IL-4)-producing CD4(+) T-cell frequencies increased significantly after vaccination in 53% of patients. Prevaccine expression of H1N1-induced HLA-DR and CD86 on B cells was high in patients who seroconverted. Seroconversion against H1N1 was strongly associated with HLA-DR expression on B cells, which was dependent on the increase between prevaccine and postvaccine H1N1-specific IL-4(+)CD4(+) T cells ($R(2) = 0.35$). High doses of MMF (2 g/d) led to lower seroconversion rates, smaller increase in H1N1-specific IL-4(+)CD4(+) T cells, and reduced HLA-DR expression on B cells. The mathematical model incorporating a MMF-inhibited positive feedback loop between H1N1-specific IL-4(+)CD4(+) T cells and HLA-DR expression on B cells captured seroconversion with high specificity. CONCLUSIONS: Seroconversion is associated with influenza-specific T-helper 2 and B-cell activation and seems to be modulated by MMF.

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Effect of Immunosuppression on Th2 and B-cell Responses to Influenza Vaccination

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Abstract

Background. Influenza vaccine immunogenicity is suboptimal in immunocompromised patients. However, there are limited data on the interplay of T- and B- cell responses to vaccination with simultaneous immunosuppression.

Methods. We collected peripheral blood mononuclear cells from transplant recipients pre and one-month post seasonal influenza vaccination. Pre- and post-vaccination, H1N1-specific T- and B-cell activation were quantified using flow cytometry. We further developed a mathematical model using T- and B-cell markers, and mycophenolate mofetil (MMF) dosage.

Results. In the 47 patients analyzed, seroconversion to H1N1-antigen was 34%. H1N1-specific IL-4-producing CD4+ T-cell frequencies significantly increased after vaccination in 53% of the patients. Pre-vaccination expression of H1N1-induced HLA-DR and CD86 on B-cells was high in patients that seroconverted. Seroconversion against H1N1 was strongly associated with HLA-DR-expression on B-cells, which was dependent on the increase of H1N1-specific IL-4+CD4+ T-cells pre-to-post vaccine (R^2=0.35). High doses of MMF ≥2g/d led to lower seroconversion rates, lower increase of H1N1-specific IL-4+CD4+ T-cells and reduced HLA-DR-expression on B-cells. The mathematical model incorporating a MMF-inhibited positive feedback loop between H1N1-specific IL-4+CD4+ T-cells and HLA-DR-expression on B-cells captured seroconversion with high specificity.

Conclusions. Seroconversion is associated with influenza-specific Th2 and B-cell activation and appears to be modulated by MMF.

Introduction:

Infection with influenza viruses in organ transplant recipients is associated with greater risk of hospitalization and mortality [1, 2]. The primary means to prevent influenza in this population is through annual influenza vaccination [3]. In immunogenicity studies, transplant recipients show suboptimal seroconversion rates against influenza [4, 5]. Factors associated with reduced humoral immunity include the use of mycophenolate mofetil (MMF), vaccination within the first year after transplantation, and receipt of a lung transplant [4, 6-9]. In particular, MMF dosage above 2g/day is a strong predictor of vaccine failure [9-11].

Protective immunity against influenza involves a multitude of interactions between the innate and adaptive immune system [12, 13]. Neutralizing antibody against influenza is well-known to protect against infection [14]. Seroprotection and seroconversion are frequently used as surrogates of vaccine protection in the general population. Although neutralizing antibodies play an important role in prevention of infection, influenza-specific T-cell responses add to the protection and may be cross-reactive to provide broad protection against drifted strains of influenza [15]. While Th1 and cytotoxic T-cell response clear viral infected cells, Th2 responses stimulate antibody production. The type of immunity stimulated by influenza vaccination likely defines the degree of protection.

Previous studies of cellular immunity against influenza after transplantation have focused on interferon-gamma (IFN-γ, a Th1 cytokine) production from T-cells and have not shown an association with humoral immunity [16, 17]. Limited data exist for the transplant population and the interaction of influenza-specific Th2-cells and B-cells.

We hypothesized that influenza vaccine immunogenicity after transplantation is likely to be related to the interplay of Th2 and B-cells and that immunosuppression is an important regulator. To test these hypotheses, we analyzed humoral and cellular immunity in a cohort of solid organ transplant patients and healthy controls that were given influenza vaccine. Peripheral blood mononuclear cells were stimulated with influenza-antigen and underwent flow-cytometric analysis for intracellular IFN-γ as a marker for Th1-response, IL-4 as a marker of Th2-response and HLA-DR and CD86-expression as activation markers for B-cell response. In addition, we developed a computational model to investigate the influence of MMF on the interplay of T- and B-cells.

Materials and Methods

Patient population

From the original trial comparing intradermal vs. intramuscular influenza vaccine, adult solid organ transplant patients were randomly selected to participate in a cellular immunity substudy (n=47) [6]. No significant differences in humoral immunity between intradermal and intramuscular vaccine were seen [6]. All patients received non-adjuvanted 2010-11 influenza vaccine containing the following influenza strains: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008 either in a high-dose (18 µg per antigen) intradermal (Intanza, Sanofi-Pasteur) or a standard dose (15 µg per antigen) intramuscular preparation (Vaxigrip, Sanofi-Pasteur). Patients had whole blood collected pre-vaccination and 4 weeks post-vaccination.

Eleven healthy volunteers (HV) were separately recruited, and received standard intramuscular vaccination with the 2011-12 vaccine (Vaxigrip, Sanofi-Pasteur). The

vaccine viruses in the 2011-12 vaccine were identical to those in the 2010-11 vaccine. The study protocols were approved through the institutional research ethics board and written informed consent was obtained from all participants.

Humoral Response to Vaccine

Sera underwent hemagglutination inhibition assay (HAI) for the H1N1-antigen contained in the vaccine as previously described [18]. Definitions of vaccine immunogenicity were based on recommendations for annual licensure of influenza vaccine (European Medicines Agency, document: CHMP/VWP/164653/2005). Seroconversion was defined as a ≥4-fold rise in titer from pre-vaccination and a post-vaccine titer ≥1:40. However, if pre-vaccination antibodies are present, the definition of 'seroconversion' may also represent a booster effect or a cross-reactive immune response. Seroprotection was defined as a titer of ≥1:40.

PBMC isolation and stimulation

Peripheral blood mononuclear cells were isolated from whole blood using a Ficoll-based method and cryopreserved until use. PBMCs were stimulated with formalin inactivated, partially purified A/California/7/2009 (H1N1) (NIBSC, NYMC-X179A, UK), the same strain as in the vaccine. A final concentration of 0.3µg/mL was used to stimulate cells for 16h at 5% CO_2. Cells were stimulated in RPMI containing 5% fetal calf serum, and 1% Glutamax (R5, all Sigma) at a concentration of 2 x 10^5 cells per well.

Flow cytometry

Experiments were performed on a FACSCanto II (BD, Franklin Lakes, NJ) and analyzed with FlowJo v 10.0.5 (TreeStar, Ashland, OR). Figure S1 shows the overall gating strategy (**Figure S1**). Media alone served as negative control and was subtracted from samples stimulated with influenza-antigen. LIVE/DEAD® staining was performed (near-IR; Invitrogen). Markers for identifying T-cell subsets were CD3 (Pacific Blue), CD4 (PE-Cy7), and CD8 (characterized as the CD4 negative gate). Intracellular cytokine staining was performed according to previously published protocols [19]. IFN-γ (Alexa Fluor 488) and IL-4 (APC) were used as key representatives for Th1 and Th2 cytokine production respectively. All reagents including perm and fixation buffers and antibodies were from eBioscience (San Diego, Ca). Isotype controls have previously been used to establish the assays.

Markers for B-cell subsets were CD20 (Alexa Fluor 488) and CD27 (PE), where naïve B-cells are $CD20^{+}CD27^{-}$ and memory B-cells are $CD20^{+}CD27^{+}$. HLA-DR (Pacific Blue) and CD86 (APC) served as activation markers (Biolegend – San Diego, CA - or eBioscience).

Cytokine profile

Cell-free supernatants from H1N1-stimulated PBMCs were collected after 24h and used for cytokine analysis. A 17-plex luminex-based cytokine profiling kit was used (Eve Technologies, Calgary, Canada): Fractalkine, IFN-α, IFN-γ, GRO, MCP-3, IL-13, sCD40-L, IL-9, IL-1β, IL-2, IL-4, IL-5, IL-6, IP-10, MCP-1, MIP-1α, and TNF-α.

Cytokine profile analysis

GeneSpring GX version 12 (Agilent Technologies, Santa Clara, CA) was used for cluster and principal component analysis (PCA) of the cytokines measured in H1N1-stimulated PBMCs. Non-stimulated samples were subtracted from stimulated PBMCs. Percentile shift was used as normalization algorithm and baseline transformation was performed to the median of all samples. Hierarchical clustering was done using Euclidean as similarity measure and Centroid as linkage rule. PCA was used to detect major trends in the experimental conditions. ANOVA cluster analysis was used to compare antibody response clusters.

Mathematical model

We constructed a phenomenological ordinary differential equation (ODE) model as described in detail in **Supplementary Information and Table S1**. Numerical simulations were performed in MATLAB (R2014a, The Mathworks, Natick, MA), with the CVODE ODE integrator from the sundialsTB toolbox [20]. Parameter estimation was performed using the MEIGO toolbox [21], and confidence intervals were determined using the HYPERSPACE toolbox [22].

Statistical analysis

Statistical analyses were performed using SPSS Statistics (version 20.0, IBM, Chicago, IL) and GraphPad Prism (version 4.0, GraphPad Software, La Jolla, CA). Data are shown with median and inter-quartile ranges. Categorical variables were analyzed using a Chi-Square (Chi^2) test. Continuous non-normal distributed data (Shapiro Wilk test) were

analyzed using a Mann-Whitney U test (MWU) or if paired using a Wilcoxon matched pairs rank test (WCR). All tests were two-tailed.

Results:

Humoral immunity is reduced by mycophenolate mofetil

We enrolled 51 transplant recipients for cellular immune assays. Of these, two were lost to follow-up and two patients did not have sufficient T-cells in the post-vaccination sample for analysis. Of the remaining 47 patients, the median age was 53.4 years (range 21-77) and most patients (51%) were kidney transplant recipients (**Table 1**). The majority of patients were on a combination of immunosuppressing drugs. All transplant patients had received prior year influenza vaccine and did not previously have microbiologically-proven influenza infection. By HAI, the pre-vaccine seroprotection rate to influenza A/H1N1 was 46.8% (22/47). Post-vaccine the seroprotection rate was 68.1% (32/47) to influenza A/H1N1, but only 16/47 (34%) demonstrated seroconversion. Transplant recipients on ≥2g/d MMF showed significantly lower geometric mean antibody titers than those on <2g/d (GMT 43.1 vs 128.4; p=0.034). The median trough level of tacrolimus and cyclosporine in patients on ≥2g/d MMF was not statistically significant different compared to those on <2g/d (7.85µg/mL vs. 6.5µg/mL; 68µg/mL vs. 187µg/mL respectively). In lung transplant recipients vs. non-lung transplant recipients seroconversion rates were not significantly different for H1N1, p=0.353.

H1N1-specific IL4-producing CD4+ T-cells, but not IFNγ-producing CD4+ or CD8+ T-cells are associated with seroconversion after H1N1 vaccination

In transplant patients, H1N1-specific IL-4+CD4+ T-cells showed a significant increase pre- to post-vaccination (median 0.32% to 0.78%; p=0.04) (**Figure S2A**). Of the 47 patients, 25/47 (53.2%) had an increase in H1N1-specific IL-4+CD4+ T-cells post-vaccination. This increase was driven primarily by seroconverting patients. We observed a significant induction of post-vaccination IL-4+CD4+ T-cells only in persons with seroconversion (**Figure 1A**). Patients who were receiving ≥2g/d MMF had a lower, non-significant increase in their IL-4+CD4+ T-cells, whereas those on lower MMF doses had a significant increase (p=0.035; **Figure 1B**). The frequency of H1N1-specific IFNγ+CD4+ and IFNγ+CD8+ T-cells did not show a significant increase post-vaccination (**Figure S2B**). IFNγ+ T-cell frequencies were not significantly associated with seroconversion and did not show any association with MMF dose (data not shown). However, the pre-to-post vaccination ratio of IL-4+CD4+ to IFNγ+CD4+ T-cells was significantly higher in patients with seroconversion, suggesting differences in expansion dynamics of T-cell subsets (median 1.7-fold to 1.0-fold, p=0.047). Lung-transplant recipients showed a median pre-vaccination IL4+CD4+ T-cell frequency of 0.33% vs. 0.35% in non-lung transplants (p=0.26). Similarly, post-vaccination IL4+CD4+ T-cell frequency was 0.62% vs. 0.86% (p=0.58) in lung transplant vs. other transplant types.

Expression levels of H1N1-induced CD86 and HLA-DR on B-cells predicts seroconversion following H1N1 vaccination

We measured B-cell activation markers after stimulation with H1N1-antigen. In

transplant recipients, HLA-DR-expression in all B-cells (as measured by mean fluorescence intensity) did not significantly increase after vaccination. However, baseline HLA-DR-expression was significantly greater prior to vaccination in patients that eventually went on to seroconversion. This was true for both the naïve B-cells subset (CD20+CD27-, p=0.0002) as well as memory B-cells (CD20+CD27+, p=0.0003) (**Figure 2A**). Table 2 provides an overview comparison of patients in different subsets (**Table 2**).

H1N1-inducible CD86-expression did not significantly change after vaccination. However, CD86-expression was significantly greater in patients that seroconverted specifically in the naïve B-cell subset **(Figure 2B**). In addition, post-vaccination, patients receiving ≥2g/d MMF had reduced H1N1-stimulated CD86-expression on naïve B-cells compared to those receiving <2g/d (p=0.05) (**Figure 2C**). Pre-vaccination (baseline) levels of CD86- and HLA-DR-expression were predictors of seroconversion (AUC for HLA-DR 0.843). For HLA-DR (CD86) expression, the highest sensitivity and specificity were 76.5% (70.6%) and 84.6% (76.9%) , respectively (**Table 3**). We further explored the role of IL-4+CD4+ T-cells on B-cell activation, a process that is partially regulated by T-helper cells and Th2-cytokines such as IL-4. In patients who seroconverted, the increase in H1N1-specific IL-4+CD4+ T-cells post-vaccination was directly correlated to HLA-DR-expression on B-cells (p=0.019; **Figure 2D**).

H1N1-induced cytokine profile pattern reflects seroconversion to influenza in transplant patients

Cytokine profiles were measured in supernatants collected from post-vaccination samples

in which PBMCs were stimulated with H1N1-antigen. We explored the differences in cytokine profiles with and without seroconversion using a heat-map and principal component analysis (**Figure 3A)**. Based on the expression profile of 28 immune markers (including 17 cytokines), the cluster analysis indicated three predominant groups. These three clusters represent: (1) patients without seroconversion but with seroprotection, (2) patients without seroconversion or seroprotection (non-responders), and (3) patients with seroconversion and seroprotection. **Figure 3B** shows the mean expression of each marker in the three clusters. Cluster (1) showed a relatively high expression of Th2-cytokines, and low expression of Th1-cytokines; this was associated with high pre- and post-vaccine HAI antibody titers to H1N1 even though these patients did not seroconvert. Cluster (3) identified a population of patients with a significant increase in HAI titers after vaccination; in particular the B-cell activation markers were significantly upregulated in these patients. A two dimensional PCA indicated that HLA-DR-expression and Th2-cytokines may be responsible for this clustering (data not shown).

A phenomenological mathematical model predicts vaccine response

We developed a dynamic mathematical model of the following structure (**Figure 4A**): An assumption for the model was that IL4-producing CD4+ T cells activate B cells and upregulate HLA-DR expression in a positive feedback loop. This interaction results in antibody production from B-cells. MMF serves as a dose-dependent inhibitor of this feedback loop. The model describes the evolution over time of the percentage of H1N1-specific IL-4+CD4+ T-cells, HLA-DR-expression on B-cells, and serum antibody (HAI) titers as a result of H1N1 vaccination.

Using this model, day 30 (post-vaccination) values for each variable as well as these variables' dynamics after vaccination can be predicted in patients who seroconverted vs. those that did not seroconvert. The model was calibrated to conservatively predict seroconversion, and based on our current data it achieves a sensitivity of 70% and specificity of 100% as determined by leave-one-out cross validation (for details see **Supplement Information** and for estimated model parameter **Table S1**). **Figure 4B** shows the predicted dynamics of the IL-4+CD4+ T-cell frequency, HLA-DR-expression on B-cells, and H1N1 antibody HAI titer starting from patient baseline values at day 0 with modulation by actual MMF dosages. These simulation results illustrate a strong dependency of seroconversion on sufficient pre-vaccination HLA-DR-expression levels. Simulations with hypothetical low (**Figure 4C**) and high (**Figure 4D**) daily MMF demonstrate an inhibitory effect of MMF that is especially pronounced at high dosages (≥ 2g/d MMF).

Healthy Volunteer Response to Vaccine

Humoral and cellular vaccine responses were also evaluated in 11 healthy volunteers. Healthy volunteers were significantly younger than the transplant recipients and they did not receive immunosuppressive drugs. All healthy volunteers had received influenza vaccine in prior years and did not have a history of microbiologically-proven influenza infection. A significant increase in HAI titers to H1N1 after immunization (GMT 66 to 345; p=0.009) was observed (100% seroprotection and seroconversion to H1N1). Compared to transplant patients, geometric mean titers were significantly higher in healthy controls (83 vs. 345; p=0.016). Similar to transplant recipients, healthy volunteers

also showed a significant increase in the frequency of H1N1-specific IL-4+CD4+ T-cells after vaccination. However, unlike transplant recipients, healthy volunteers did show an increase in IFNγ+CD4+ T-cells post-vaccination (**Figure S2B**). H1N1-induced B-cell activation markers were high in healthy volunteers and were similar to transplant recipients (data not shown); healthy volunteers also had no significant changes in HLA-DR- and CD86-expression pre- to post-vaccination (data not shown). The post-vaccination cytokine profile showed that healthy controls had significantly greater levels of Fractalkine, IFN-γ, MCP-3, IL-1β, IL-6, and MIP-1α (**Figure S2C**). The results of the cytokine profile are shown in Table S2. These findings should be interpreted in the context of the significantly younger age of the healthy volunteers compared to the transplant recipients.

Discussion

We performed a detailed analysis of H1N1-induced B- and T-cell responses to assess factors associated with successful seroconversion after influenza vaccination in the organ transplant population. We show that Th2-cytokines are associated with H1N1-induced B-cell activation in terms of HLA-DR (antigen presentation) and CD86 (co-stimulatory signaling) expression, as well as antibody secretion. MMF reduced IL-4+CD4+ T-cell frequencies and B-cell activation. The type of graft (lung vs. nonlung transplant) did not have an effect on T-cell responses. We employed several analytical techniques to formulate sensitive and specific predictive models to discriminate vaccine responders from non-responders.

The influenza vaccine stimulates the Th1- and Th2-pathway in order for B-cell differentiation to occur [23]. One of our key findings was the increase in H1N1-specific IL-4+CD4+ T-cells after vaccination. IL-4 is involved in humoral immunity and previously has not been explored in the context of vaccination of transplant recipients. IL-4 (originally termed B-cell stimulatory factor 1) is a cytokine produced by Th2-cells and has been shown to increase HLA-DR-expression on resting B-cells, thereby increasing production of IgG [24, 25]. Th2-responses have also been shown to be important in mice immunized with a universal influenza vaccine [26].

We did not observe a significant rise in IFNγ+CD8+ T-cell frequency after vaccination in transplant recipients, contrary to that seen in the healthy volunteer cohort. The frequency of H1N1-specific IFNγ+CD4+ and IFNγ+CD8 T-cells was consistent with previous studies of influenza vaccine [27]. These results were also similar to results shown by Cowan et al. who showed a significantly greater increase in IFNγ-responses in healthy controls compared to kidney transplant patients [28]. Another study in lung transplant recipients also did not show an increase in influenza-vaccine related IFNγ post-influenza vaccination [17]. Previous studies in transplant recipients have not found a relationship between IFNγ and humoral responses. For example, in a kidney transplant cohort, although an increase in IFNγ was seen post-vaccination, this was not associated with humoral responses [16]. The study by Cowan et al. also did not show a correlation between IFNγ T-cell responses and influenza-specific IgG responses [28]. Virus-specific CD8+ T-cells typically recognize peptides derived from internal components of the virus [29]. Thus, during influenza replication, cytotoxic T-cells could respond to a broader

spectrum of possible expressed epitopes and proteins in comparison to strain-specific antibodies to the viral HA and NA glycoproteins [30]. However, inactive vaccines do not replicate and therefore they contain only a limited spectrum of proteins, namely HA and NA glycoproteins; the induced CD8+ T-cell response may not directly correlate with an antibody response. Nevertheless, if an IFN-γ response develops, it could promote the induction of HA-specific neutralizing antibodies and may in fact help with broadening responses to heterologous influenza viruses [31, 32].

In the non-transplant literature, the ratio of Th2:Th1-cytokines has been suggested to be associated with vaccine responses in the elderly. Similar to our study, McElhaney et al. measured IL-10 as a marker of Th2 responses and showed that there was a significant rise in IL-10 post-vaccination in PBMCs of elderly persons stimulated with H1N1 [33]. In our cohort we observed a significant increase of IL-4-producing CD4+ T-cells in patients with seroconversion. In addition, our cytokine profile indicated significant differences in Th2 cytokines in patients with seroconversion and seroprotection compared to non-responding patients.

We also explored B-cell immunity using HLA-DR and CD86 as markers of activation. HLA-DR acts as a ligand for the T-cell receptor resulting in antigen presentation and further stimulation of T-cells. Additionally, CD86 acts as a co-stimulatory molecule on B-cells and interacts with CD28 on T-cells [34]. These markers have not previously been explored in the context of immunization of organ transplant recipients. The pre-vaccination expression of these markers was high in patients that seroconverted,

suggesting a role for these markers in prediction of seroconversion. Indeed, a ROC curve analysis showed that baseline HLA-DR and CD86-expression had high sensitivity and specificity in predicting seroconversion. The high responsiveness of memory B-cell subsets prior to vaccination may be partially explained by prior illness or previous vaccine. All transplant recipients and healthy volunteers had previously received influenza vaccine.

General effects on IgG serum concentration in transplant recipients treated with MMF have been previously described [35]. Several studies have now shown that high doses of MMF reduce the immunogenicity of influenza vaccination [9-11]. Our cohort also had reduced responses by HAI in patients receiving doses of ≥2g/d. MMF and MPA have been shown to inhibit B-cell activation and proliferation and plasma cell formation [36, 37]. Our study provides insight into a potential mechanism by which this occurs. We found that high doses of MMF had a deleterious impact on IL-4+CD4+ T-cell frequencies and was associated with reduced HLA-DR-expression on B-cells. Our results are consistent with a previous study which showed a dose-dependent reduction of HLA-DR-expression on B cells with increasing mycophenolate [38]. In addition, the ODE model predicts high, sustained antibody titers in most patients who seroconverted, and low antibody titers (with possibly high transient titers) in patients who did not. Without MMF, the model predicts that most patients that did not seroconvert in the study would develop high antibody titers at day 30, and with a hypothetical 3g/d MMF, the model predicts that none of the patients seroconvert. Large uncertainties in estimated model parameters, however, indicate that more data (patients and time points) and possibly

model extensions will be required to obtain final conclusions on the modeling approach's predictive power.

Our study has some limitations. Almost all our patients and healthy volunteers had been previously vaccinated and this may have influenced the response seen. We only used certain signature cytokines for the Th1 and Th2 response. It is possible that other cytokines may behave differently. In addition, although the trivalent influenza vaccine contains two A strains and one B strain, we used influenza A/H1N1 as a model for vaccine responses. It is possible that other strains of influenza such as A/H3N2 and B strains induce differing Th1 and Th2 profiles. We also had different organ types represented in our population, though this allowed us to provide a broad overview of cellular immunity to vaccine in the transplant population. An important point is that our healthy control group was also significantly younger compared to the transplant cohort. It has been well described that aging is an important factor for reduced humoral responses [33], and the comparative immunologic responses between transplant and healthy individuals should be interpreted in this context.

In summary, Th2 responses appear to be key regulators of influenza vaccine response in transplant patients. B-cell activation markers prior to immunization have the potential to predict future humoral responses to vaccine. Mycophenolate mofetil is a key regulator of these responses at the cellular level. Tailoring immunosuppression to influence the vaccine response via upregulation of a Th2 cytokine profile may be a future strategy to improve outcomes of vaccination.

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Conflict of interest: The authors of this manuscript have conflicts of interest to disclose as D.K. has received research grant from Roche and GSK and A.H. has received research grant from Roche. The other authors have no conflict of interest.

Figure Legends

Figure 1. H1N1-specific IL-4+CD4+ T-cell dynamics during vaccination

(A) Frequency of H1N1-specific IL-4+CD4+ T-cells in patients that seroconverted vs. those who showed no seroconversion to influenza A/H1N1 (n=15 vs. 32). Patients that seroconverted had a greater rise in the frequency of IL-4+CD4+ T-cells after vaccination (p=0.04).

(B) Frequency of H1N1-specific IL-4+CD4+ T-cells in patients who received ≥2g daily dose of MMF vs. those who received <2g daily dose of MMF (n=19 vs. 28).

Figure 2: H1N1-induced B-cell activation during vaccination.

The background (non-stimulated) expression of HLA DR and CD86 was subtracted. The median background for HLA DR was 95.9 A.U., and for CD86 43.7 A.U. (also see Table 2)

(A) Expression of H1N1-induced HLA-DR-expression on naïve and memory B-cells prior to vaccination is greater in patients that seroconverted vs. those who showed no seroconversion to influenza A/H1N1 (n=15 vs. 32). Bars indicate median values, whiskers show the interquartile range. Mann-Whitney U testing was used to determine significant differences.

(B) Expression of H1N1-induced CD86-expression on naïve and memory B-cells is greater in patients that seroconverted vs. those who showed no seroconversion to influenza A/H1N1 (n=15 vs. 32). Bars indicate median values, whiskers show the

interquartile range. Mann-Whitney U testing was used to determine significant differences.

(C) Expression of H1N1-induced CD86-expression on naïve B-cells post-vaccination based on MMF dose. Patients receiving ≥2g MMF daily dose had significantly less expression of CD86. Bars indicate median values, whiskers show the interquartile range. Mann-Whitney U testing was used to determine significant differences.

(D) Regression analysis of fold-change in IL-4+ T-cell frequencies and their impact on HLA-DR-expression on B-cells in patients with seroconversion (n=15).

FIGURE 3: H1N1-induced cytokine profile.

(A) Heatmap of cytokines, antibody titers and B-cell activation markers in H1N1-stimulated PBMCs of the total post-vaccine transplant cohort. Blue represents seroconversion (n=15) and red represents no seroconversion (n=28, for 4 samples no cytokine profile was available). Non-stimulated background samples were subtracted prior to normalization algorithm. Relative changes of a marker are indicated by change of color code (blue, max 5.8 fold down-regulation; red, max 5.8 up-regulation). Markers analyzed (top to bottom): FRAK, IL13, IL9, IL5, CD4 IFNγ, CD4 IL-4, IL-4, mB CD69, IL1β, GRO, sCD40L, H1N1 Antibody titer, IFNα, IL2, IFNγ, TNFα, IL6, MIP1α, MCP3, nB CD69, IP10, mB CD86, nB CD86, mo CD86, nB DR, mB DR, mo DR, MCP1. B, B-cell; mo, monocyte; CD4, CD4 T-cells. N, naïve phenotype; m, memory phenotype. DR, HLA-DR-expression.

(B) Clusters that represent (1) patients without seroconversion but seroprotection (left), (2) patients without seroconversion or seroprotection (middle), and (3) patients with

seroconversion (right). Relative changes are indicated by changes of color code (see Fig. 3A). Markers analyzed (top to bottom): FRAK, mo DR, IL13, CD4 IFNγ, IL9, IL5, CD4 IL-4, IL1β, IL-4, mB CD69, sCD40L, H1N1 antibody titer, GRO, IFNα, IFNγ, IL6, MIP1α, MCP3, IL2, TNFα, mo CD86, nB CD86, mB CD86, nB CD69, MCP1, nB DR, mB DR, IP10. B, B-cell; mo, monocyte; CD4, CD4 T-cells. N, naïve phenotype; m, memory phenotype. DR, HLA-DR-expression. A One-Way ANOVA applying the GeneSpring® software was calculated to indicate that a cytokine is differentially expressed in at least one of the three groups analyzed. The ANOVA analysis used the mean of the sum of squared deviates as an aggregate measure of variability for the selected group of genes, applying parametric testing for equal group sizes and independent samples, without post-hoc testing. PC1, PC2, and PC3 had a weight of 54.6%, 31.9% and 13.5% respectively.

FIGURE 4: Mathematical Model

(A) Overview of the structure of the phenomenological mathematical model: IL-4-producing T-cells and HLA-DR-expressing B-Cells activate each other in a mutual feedback loop that is inhibited by MMF. Antibody production is modeled in a HLA-DR-expression-dependent manner.

(B-D) Dynamics of the ODE model for IL-4+T-cell frequency, HLA-DR-expression on naïve B-cells and H1N1 antibody HAI titer starting from patient baseline values at day zero for non-seroprotected patients, distinguishing between patients who seroconverted (black) and those who did not seroconvert (red). Simulations were performed with

patient-specific MMF dosage **(B)** and two hypothetical situations where patients receive no MMF **(C)**, or a 3 g daily dose of MMF **(D)**.

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Figure 1.

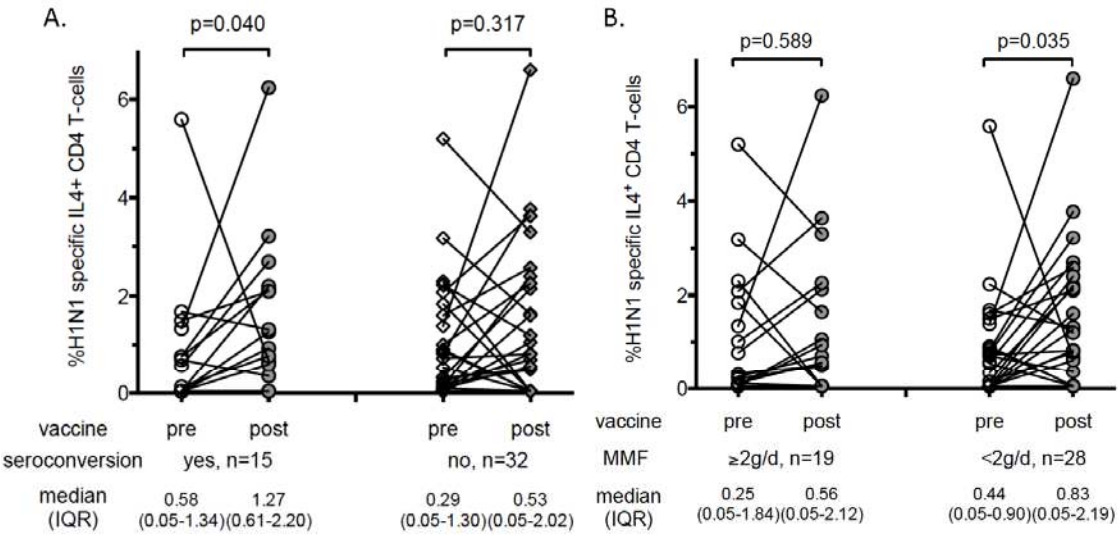

Figure 2.

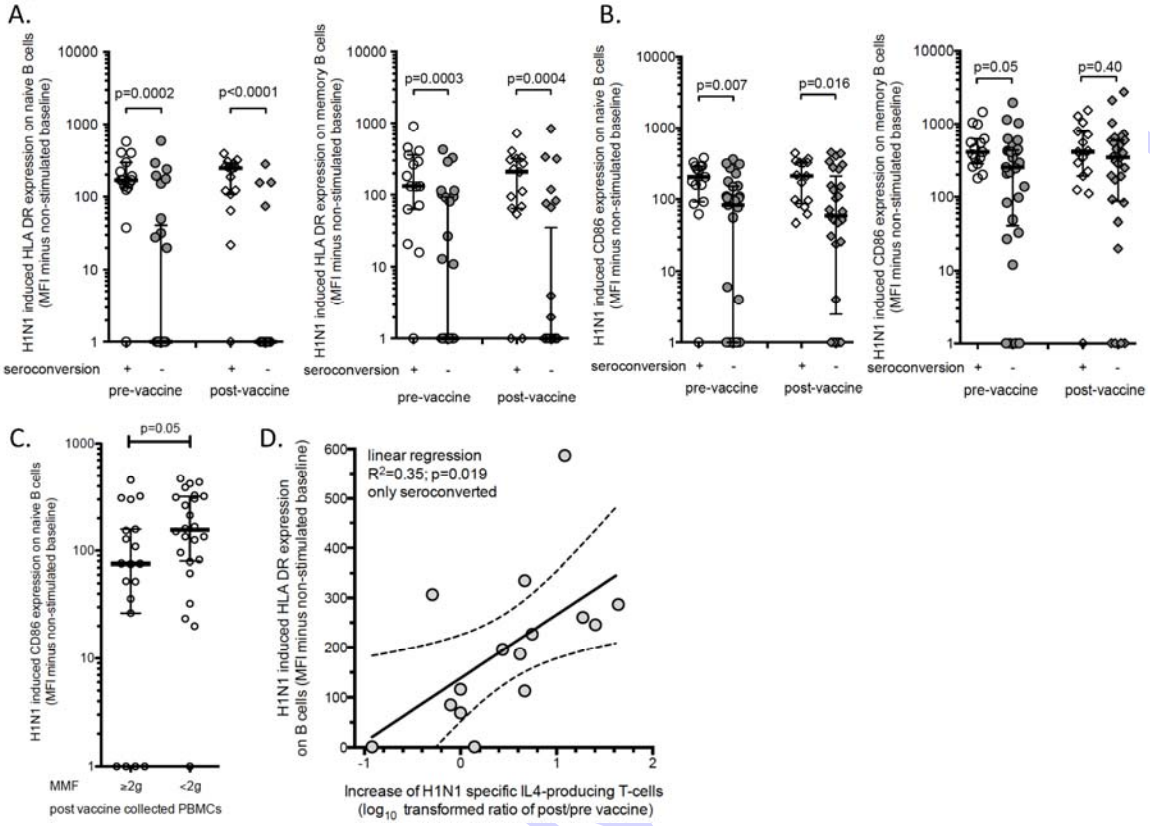

Figure 3.

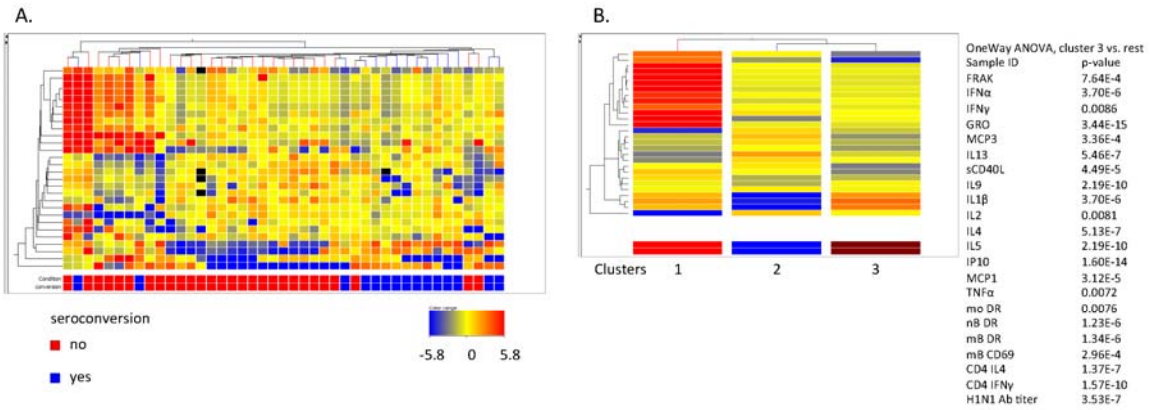

Figure 4.

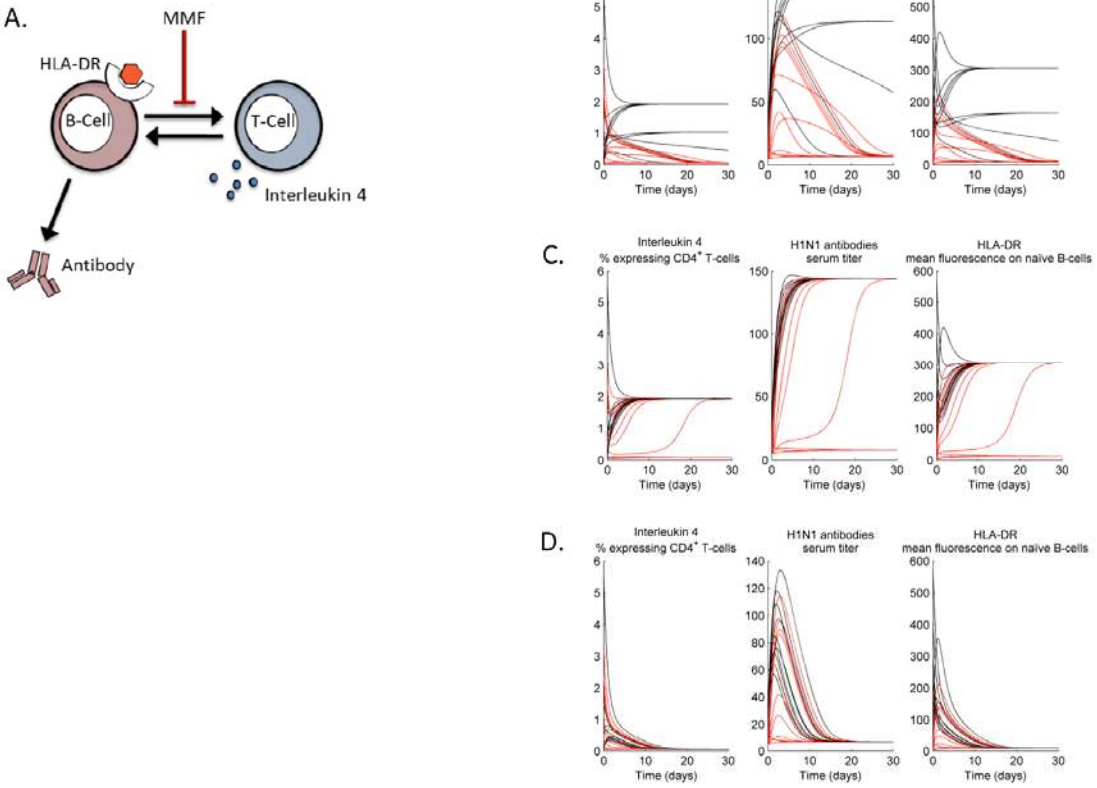

Table 1: Characteristics of transplant patients and healthy volunteers.

	Transplant recipients (n=47)	**Healthy Volunteers (n=11)**	**p-value**
Age, median (IQR) in years	53.4 (16)	33 (10)	p<0.05
Gender (m/f)	33/14 (70.2%/29.8%)	7/4 (63.6%/36.4%)	n.s.
Time post-transplant to immunization (years); median (min - max)	4.34 (0.26 – 22.34)	N/A	
Type of Vaccine			
Standard Intramuscular	26 (55%)	11 (100%)	
High-dose Intradermal	21 (44.7%)		
Seroconversion (n, %)			
Influenza A/H1N1	16 (34.0%)	11 (100%)	p<0.05
Influenza A/H3N2	12 (25.5%)	11 (100%)	p<0.05
Influenza B	7 (14.9%)	7 (63.6%)	p<0.05
Pre-vaccine Titer (GMT)			
Influenza A/H1N1	26	66	0.058
Influenza A/H3N2	19	55	0.012
Influenza B	16	12	n.s.
Post-vaccine Titer (GMT)			
Influenza A/H1N1	82	345	p= 0.016
Influenza A/H3N2	43	206	p= 0.001
Influenza B	29	34	n.s.

Type of graft (n, %)			
Kidney	24 (51.1%)	-	-
Lung	15 (31.9%)	-	-
Heart	4 (8.5%)	-	-
Liver	4 (8.5%)	-	-
Immunosuppression (n, %, median dose/d)			
Prednisone (median dose)	35 (74.5%); 5mg/d	-	-
Tacrolimus (median trough level)	36 (76.6%); 7.4μg/mL	-	-
Cyclosporin(median trough level)	9 (19.1%); 78μg/mL	-	-
MMF (median dose)	34 (72.3%); 2000mg/d	-	-
Sirolimus(median trough level)	5 (10.6%); 8.2μg/mL	-	-

GMT Geometric Mean Titer, IQR Interquartile range, MMF mycophenolate mofetil.

Table 2. B-cell activation markers before and after vaccination in B-cell subsets according to seroconversion status.

	HLA-DR (MFI)				**CD86 (MFI)**			
	seroconversion		**No seroconversion**		**seroconversion**		**No serconversion**	
Vaccine	**Pre-**	**Post-**	**Pre-**	**Post-**	**Pre-**	**Post-**	**Pre-**	**Post-**
All (CD20+)	203 (125-321)	196 (85-287)	1 (1-81)	1 (1-1)	276 (145-330)	235 (101-419)	105 (1-213)	142 (64-274)
Naïve (CD20+CD27-)	168 (133-296)	246 (108-287)	1 (1-41)	1 (1-1)	204 (92-289)	211 (87-328)	83 (1-151)	59 (3-209)
Memory (CD20+CD27+)	132 (63-358)	208 (65-318)	1 (1-94)	1 (1-36)	409 (282-623)	414 (192-790)	253 (41-436)	348 (88-596)

Mean fluorescence intensity (MFI) values are shown in pH1N1 overnight stimulated B-cell subsets (background subtracted).

Table 3: Receiver operating characteristic curve statistics of HLA-DR and CD86 expression on all B-cells pre-vaccination for the prediction of successful seroconversion.

Variable	AUC	Standard error	p-value	95% CI	Best Cut off value (MFI)	Sensitivity	Specificity
HLA-DR	0.843	.060	<0.0001	0.72-0.97	123	76.5%	84.6%
CD86	0.700	.083	0.011	0.58-0.89	213	70.6%	76.9%

AUC area under the curve